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Introduction

Activation of the complement system is an important component of host defense. Following infection, triggering of the complement activation cascade via direct binding of complement components to microbial surfaces may lead to opsonization and pathogen elimination via humoral and cellular mechanisms. Furthermore, complement activation may trigger and amplify the acquired immune system. Until now, three different pathways of complement activation have been described, i.e. the classical pathway, the alternative pathway, and the lectin pathway. These pathways converge at the level of C3, leading to activation of the common terminal complement pathway and finally formation of the membrane attack complex (reviewed in (Walport, 2001a; Walport, 2001b)).

Defects in the complement system may lead to a partial or complete blockade of the complement activation cascade. Depending on the level of the defect, either the induction phase or the effector phase of complement activation may be hampered, and the defect may affect more than one pathway. Impaired function of the complement system may occur due to genetic defects, or due to acquired deficiencies of complement components. Acquired complement deficiencies may occur due to formation of autoantibodies to complement components or due to excessive complement consumption (Walport, 2001a; Walport, 2001b; Trouw et al., 2001). Genetic complement deficiencies have been described at all levels of the system, i.e., in the classical pathway, in the alternative pathway, in the lectin pathway, and in the terminal pathway from C3 until C9 (Crawford and Alper, 2000).

Most complement defects are associated with disease, ranging from a relatively mild increase in the susceptibility to infections to the occurrence of a severe systemic autoimmune syndrome. Furthermore, impaired complement function is associated with the occurrence of flares in patients with systemic lupus erythematosus (SLE) (Walport, 2001a; Walport, 2001b; Crawford and Alper, 2000). Therefore, functional assays to measure complement activity in human serum have a clear diagnostic and prognostic value.

Complement function in serum is mostly measured using hemolytic assays that enable the functional assessment of the classical complement pathway and the alternative complement pathway, respectively. In these hemolytic assays, the function of the complement pathways is expressed as its ability to generate the C5b-9 complex upon activation. However, such an assay is currently not available for the evaluation of the lectin pathway of complement in serum.

The lectin pathway of complement (LP) is mainly driven by binding of mannan-binding lectin (MBL) to one of its carbohydrate ligands (Petersen et al., 2001b). Binding will induce activation of the MBL-associates serine proteases (MASP) leading to formation of the C3 convertase C4b2a (Matsushita and Fujita, 1992; Thiel et al., 1997) (Matsushita et al., 2000b). The MBL-MASP complex, being the recognition complex of the LP, has a strong structural and functional similarity to the C1 complex, the recognition unit of the classical complement pathway (CP). The C1 complex, consisting of C1q and the serine proteases C1r and C1s, is mainly activated by binding of C1q to immune complexes consisting of IgG and IgM, also leading to the generation of C4b2a.

The gene encoding human MBL is characterized by a high degree of polymorphisms, both in the promoter region and in exon 1 (Garred et al., 1996). In the promoter region, various single nucleotide polymorphisms (SNP) have been described that are involved in quantitative gene expression and hence determine the MBL plasma concentration. Furthermore, at least five different SNP's have been discovered in exon 1 of the MBL gene, encoding the collagenous region of MBL (Sumiya et al., 1991) (Lipscombe et al., 1992) (Madsen et al., 1994; Neonato et al., 1999). At codon 52 (D genotype), codon 54 (B genotype) and codon 57 (C genotype), SNP's are frequently present: the allele frequency in the Caucasian population is 5 % (D allele), 13 % (B allele) and 2 % (C allele), respectively (Garred et al., 1996). These SNP's induce amino acid substitutions that affect the polymerization of the MBL molecule in a dominant way. Accordingly, small-sized MBL

molecules are generated with impaired functional properties (Super et al., 1992; Wallis and Cheng, 1999; Wallis, 2002).

The presence of MBL-mutant alleles is associated with increased susceptibility to infections, mainly in childhood and in immune-compromised individuals (Summerfield et al., 1995; Hibberd et al., 1999; Peterslund et al., 2001; Neth et al., 2001). Furthermore, the above-described SNP's confer an increased progression of severe chronic diseases such as cystic fibrosis, rheumatoid arthritis, and SLE (Garred et al., 1999b; Graudal et al., 2000; Garred et al., 1999a). Therefore, since there is such a high inter-individual variability in expression of (functional) MBL, which is determined by multiple variables, functional assessment of LP activity in human serum generates novel and most likely clinically more relevant possibilities for risk assessment for individual patients.

We now developed an ELISA-based LP assay that enables the functional evaluation of successive steps of autologous complement activation in full human serum without any interference of the CP. Measurement of the activity of the CP and the alternative complement pathway (AP) in a similar ELISA system provides the possibility of parallel quantification of all three complement activation pathways in patient serum using one assay system.

Materials and Methods

Human materials

Human serum was obtained from 70 healthy adult volunteers and immediately frozen at -80 °C in aliquots. Genomic DNA was isolated from heparinized blood as described below. Human DNA samples with known MBL gene polymorphisms were kindly provided by Dr. P. Garred, (Copenhagen, Denmark). Outdated healthy donor plasma was obtained from the Bloodbank Leiden-Haaglanden, Leiden, the Netherlands. From a patient with Kahler's disease of the IgM type, plasma was obtained that became available after a plasmapheresis treatment.

Anti-C1q and anti-MBL antibodies

Monoclonal antibodies against C1q were produced in mice as described before (Hoekzema et al., 1988). The anti-C1q mAb 2204 (IgG1) is directed against the globular head domain of C1q and is able to inhibit the binding of C1q to IgG, as well as C1q-dependent hemolysis (Roos et al., 2001b). For the purification of mAb 2204, gamma globulins were precipitated from ascites using 50 % $(NH_4)_2SO_4$. The precipitate was dialyzed against 10 mM Tris containing 2 mM EDTA (pH7.8) and subjected to anion exchange chromatography using DEAE-Sephacel (Pharmacia, Uppsala, Sweden). Proteins were eluted using a salt gradient and the fractions that showed binding of mouse IgG to C1q-coated ELISA plates in the presence of 1 M NaCl were pooled, concentrated, dialyzed against PBS and stored at -80 °C.

Polyclonal anti-C1q antibodies were produced in rabbits. New Zealand White rabbits were immunized (weekly for four weeks) with 180 µg C1q dissolved in complete Freunds adjuvant, resulting in antisera with a positive titer on C1q-coated ELISA plates beyond 1/25,000. IgG was precipitated from rabbit serum using 40 % $(NH_4)_2SO_4$ and purified using DEAE-Sephacel as described above.

Starting from purified rabbit IgG anti-C1q, Fab fragments were generated using papain. Therefore, IgG was dialyzed against 10 mM phosphate buffer containing 10 mM L-cysteine and 2 mM EDTA (pH 7.0). Subsequently, mercuripapaine (from Sigma) was added (1 % w/w of the protein content) followed by incubation for 16 hours at 37 °C. After dialysis against PBS, the sample was applied to sepharose-coupled protein G (from Pharmacia, Uppsala, Sweden), and the fall through fractions, containing Fab fragments, were pooled, concentrated, and used for experiments. Analysis by non-reducing SDS-PAGE showed a prominent band at approximately 45 kD.

A mouse mAb directed against the lectin domain of human MBL (mAb 3F8) was kindly provided by Dr. G.L. Stahl (Harvard Medical School, Boston, Massachusetts, USA) (Collard et al., 2000).

Preparation of human C1q and C1q-depleted serum

Human C1q was isolated from human donor plasma exactly as described previously and was stored at -80 °C (Roos et al., 2001b). Isolated C1q was able to completely restore the lysis of antibody-coated erythrocytes induced by C1q-depleted human serum.

For the preparation of C1q-depleted serum, undiluted normal human EDTA plasma (obtained from a donor with the MBL/AA genotype) was applied on column consisting of Biogel A5 (from Biorad) coupled to rabbit IgG anti-human C1q. The column was washed using Veronal-buffered saline (VBS; 1.8 mM Na-5,5-diethylbarbital, 0.2 mM 5,5-diethylbarbituric acid, 145 mM NaCl) containing 10 mM EDTA. Fractions were tested in a C1q-dependent hemolytic assay in the absence or presence of purified C1q, as previously described (Roos JI). Fractions that showed complete erythrocyte lysis in the presence of C1q, but not in the absence of C1q, were pooled and concentrated until the original volume. After recalcification, C1q-depleted serum was stored at -80 °C.

Isolation of human IgM

Plasma containing an IgM paraprotein was dialyzed against 10 mM sodium acetate containing 2 mM EDTA (pH 5.0). The precipitated proteins were recovered by centrifugation, dissolved in PBS, dialyzed against Tris/EDTA buffer (10 mM Tris, 2mM EDTA, pH 7.8 and conductivity 5.0 mS), and subjected to anion exchange chromatography using DEAE-Sephacel. IgM that eluted in the salt gradient was pooled, dialyzed against 10 mM sodium acetate (6.0 mS, pH 7.0) and applied to a CM-C-50 Sephadex anionic exchange column (from Pharmacia). Following elution with a salt gradient, fractions containing IgM were pooled, concentrated, and applied to a Superdex 300 gel filtration column. Peak fractions containing IgM and free of IgG were pooled, concentrated, and stored at -80 °C.

Assessment of functional lectin pathway activity by ELISA

Functional activity of the lectin pathway was assessed by ELISA using immobilized mannan as a ligand. Mannan was obtained from Sigma (from Saccharomyces Cerevisiae; M7504), dissolved in PBS (10 mg/ml) and stored at -20 °C. Nunc Maxisorb plates (Nunc, Roskilde, Denmark) were coated with mannan (100 µg/ml) in coating buffer (100 mM Na_2CO_3/ $NaHCO_3$, pH 9.6), for 16 hours at room temperature or for 2 hours at 37 °C. After each step, plates were washed three times with PBS containing 0.05 % Tween 20. Residual binding sites were blocked by incubation with PBS containing 1 % BSA for one hour at 37 °C. Serum samples were diluted in GVB++ (VBS containing 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.05 % Tween-20, and 0.1 % gelatin; pH 7.5) in the presence of mAb 2204 (20 µg/ml) as an inhibitor of C1q, unless otherwise indicated. This mixture was pre-incubated for 15 minutes on ice, before addition to the plates. The plates were then sequentially incubated for 1 hour at 4 °C and for 1 hour at 37 °C, followed by washing.

Complement binding was detected using mouse mAb conjugated to digoxygenin (dig) using digoxygenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (from Boehringer Mannheim, Mannheim, Germany) according to instructions provided by the manufacturer. Detection of C1q, C4, C3, and C5b-9 was performed using mAb 2214 (anti-human C1q), mAb C4-4a (anti-human C4d), RFK22 (anti-human C3) and AE11 (anti-C5b-9, kindly provided by Dr. T.E. Mollnes, Oslo, Norway), respectively. Binding of mAb was detected using dig-conjugated sheep anti-mouse antibodies (Fab fragments) followed by HRP-conjugated sheep anti-dig antibodies (Fab fragments, both from Boehringer Mannheim). All detection antibodies were diluted in PBS containing 1 % BSA and 0.05 % Tween 20. Enzyme activity of HRP was detected following incubation with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (from Sigma; 2.5 mg/ml in 0.1 M Citrate/Na_2HPO_4 buffer, pH 4.2) in the presence of 0.01 % H_2O_2, for 30-60 min. at room temperature. The OD at 415 nm was measured using a microplate biokinetics reader (EL312e, from Biotek Instruments, Winooski, Vermont, USA).

Assessment of functional classical pathway activity by ELISA

The protocol for the functional activity of the classical pathway was similar to the protocol for the LP assay, as described above, with important modifications. As a ligand for CP activation, human IgM was coated at 2 µg/ml. After blocking of residual binding sites, serum samples, diluted in GVB++, were added to the plate and incubated for 1 hour at 37 °C. Complement binding was assessed using dig-conjugated mAb directed against C1q, C4, C3, and C5b-9, followed by the detection of mAb binding using HRP-conjugated sheep anti-dig antibodies.

Assessment of functional alternative pathway activity by ELISA

The protocol for the functional activity of the alternative pathway was similar to the protocol for the LP assay, as described above, with important modifications. As a ligand for AP activation,

LPS was coated at 10 µg/ml. LPS from Salmonella Typhosa was obtained from Sigma (L-6386), dissolved in PBS at 1.6 mg/ml and stored at -20 °C. Plates were blocked using 1% BSA in PBS. Serum samples were diluted in GVB/MgEGTA (VBS containing 10 mM EGTA, 5 mM $MgCl_2$, 0.05 % Tween-20, and 0.1 % gelatin; pH 7.5) and incubated in the plate for 1 hour at 37 °C. Complement binding was assessed using dig-conjugated mAb directed against C4 and C3, followed by the detection of mAb binding using HRP-conjugated sheep anti-dig antibodies.

Quantification of anti-mannan antibodies in human serum

For the quantification of anti-mannan antibodies in human serum, ELISA plates were coated with mannan and blocked with 1 % BSA in PBS. Serum samples were diluted 1/100 for detection of IgG anti-mannan Ab, 1/10 for detection of IgA anti-mannan Ab, and 1/40 for detection of IgM anti-mannan Ab, respectively, unless otherwise indicated. For quantification, pooled human IgG (48 mg/ml IgG), pooled human IgA (41 mg/ml IgA), and pooled human IgM (35 mg/ml IgM) were used as a standard for detection of IgG, IgA and IgM anti-mannan antibodies, respectively (kindly provided by Biotest Pharma GmbH, Dreieich, Germany). The concentration of anti-mannan antibodies in these preparations was arbitrarily set at 1000 U/ml. All samples were diluted in PBS containing 0.05 % Tween 20 and 1 % BSA. Antibody binding was detected using biotinylated HB43 (mouse mAb anti-human IgG), biotinylated HB57 (mouse mAb anti-human IgM) and dig-conjugated 4E8 (mouse mAb anti-human IgA), respectively, followed by either HRP-conjugated streptavidin or HRP-conjugated sheep anti-dig antibodies (both from Boehringer).

DNA isolation

Genomic DNA was isolated from heparinized blood according to standard procedures (Miller et al., 1988). Briefly, 10 ml blood was diluted with 40 ml EL buffer (erythrocyte lysis buffer: 155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH 7.4) and incubated on ice for 20

minutes. After centrifugation (10 minutes at 500 g), the pellet was washed with 25 ml EL buffer, and resuspended in 3 ml of KL buffer (10 mM Tris, 2mM EDTA, 400 mM NaCl, pH 8.4), followed by thoroughly shaking. After addition of 25 µl pronase (20 mg/ml in water, Boehringer Mannheim, Germany) and 150 µl SDS (20 % in water), the mixture was incubated in a shaking water bath at 37 °C for 18 hours. Finally, the DNA was precipitated with ethanol, dissolved in 0.5 ml TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.4), heated for 5 minutes at 65 °C, and kept at 4 °C.

PCR amplification of exon 1 of the MBL gene

Exon 1 of the MBL gene was amplified from genomic DNA by PCR. Starting from 1 µl of genomic DNA (approximately 0.7 µg), a 40 µl PCR reaction was performed, using 0.25 mM dNTP (from Pharmacia Biotech), 0.8 U Amplitaq (from Perkin Elmer, Wellesley, MA), and 12.5 pmol of both PCR primers (from Eurogentec, Seraing, Belgium; Table 1) in PCR buffer (10 mM Tris HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.6 mg/ml BSA, pH 8.3). The PCR reaction was performed in a Peltier Thermal Cycler (PTC200, from MJ Research, Waltham, MA) using the following program: denaturation for 5 min at 95°C, followed by 36 cycles of 1 min. 95 °C, 1 min. 57 °C, and 1 min. 72 °C, and a final elongation period for 7 min. at 72 °C. Evaluation of the PCR products by agarose electrophoresis showed one specific band of the expected molecular weight (679 bp) with an estimated concentration of about 30 ng/µl.

Oligonucleotide Ligation Assay for MBL genotyping

For detection of MBL mutant alleles at codon 52, 54, and 57, three different OLA protocols were developed. For each OLA, two reactions were performed in parallel, using either the wildtype or the mutant primer, both in combination with a common primer (Table 1). PCR products were first heated for 5 min at 99 °C. The OLA reaction was performed in a 20 µl reaction mixture consisting of 2 µl of PCR product, 5 pmol common primer, 5 pmol of either the wildtype or the

mutant primer (Table 1), and 1.2 U Taq DNA ligase, using the buffer supplied by the manufacturer (from New England Biolabs, Beverly, MA). The following program was run in a PTC 200 Thermal Cycler: denaturation for 2 min. at 94 °C, 10 cycles of 10 sec. 94 °C and 3 min. 60 °C, followed by a final incubation of 5 min. at 99 °C. For OLA detection of codon 57 polymorphisms, probe anealing was performed at 54 °C instead of 60 °C.

For detection of OLA products, ELISA plates were coated with avidin (20 µg/ml, from ICN Biomedicals inc., Aurora, Ohio, USA) and aspecific binding sites were blocked with PBS containing 3 % BSA. The OLA reaction mixture was 1/5 diluted in PBS containing 1 % BSA, added to the plate, and incubated for 1 hour at 37 °C. Plates were washed and dig-conjugated reaction products were detected using HRP-conjugated sheep anti-dig antibodies as described above.

Results

Anti-mannan antibodies in human serum

Mannan is a major ligand of MBL that can efficiently activate the LP of complement. However, human serum contains anti-carbohydrate antibodies, probably resulting from previous microbial contacts. Such anti-carbohydrate antibodies may bind to mannan and the resulting immune complex may contribute to complement activation by mannan via activation of the classical complement pathway (Petersen et al., 2001a; Super et al., 1990). Mannan-binding antibodies are clearly detectable in human serum as assessed by ELISA (Fig. 1). Incubation of pooled human IgG (Fig. 1A), IgA (Fig. 1B) and IgM (Fig. 1C) on immobilized mannan resulted in a dose-dependent binding of IgG, IgA, and IgM as detected by isotype-specific mAb. As a control, parallel incubations were performed on immobilized BSA, resulting in low or undetectable background binding of pooled Ig. Incubation of three sera from healthy donors on mannan-coated plates resulted in strong dose-dependent IgG binding in all three sera. In donor 1, IgA and IgM anti-mannan Ab were undetectable, serum from donor 2 contained IgG, IgA and IgM anti-mannan antibodies, whereas in donor 3 some IgM binding was observed but no IgA binding (Fig. 1A-C). Binding of Ig was undetectable following incubation of serum on BSA-coated plates (Fig. 1A-C). Quantification of anti-mannan antibodies in serum from 70 healthy donors is presented in Fig. 1D. IgG and IgM anti-mannan Ab were present in nearly all donors, with a large interindividual variation, whereas IgA anti-mannan Ab were detected in 63 % of the donors. No significant correlation was observed between the three major isotypes of anti-mannan antibodies, or between anti-mannan antibodies and MBL concentrations (not shown).

Functional characterization of the lectin pathway in the presence of C1q-inhibitory Ab

Both the LP and the CP are calcium-dependent and lead to activation of C4. A distinction between both pathways can be made by selection of a specific ligand that induces specific activation of either the LP or the CP. In view of the presence of anti-mannan Ab in human serum, mannan is likely to activate both the LP, via MBL, and the CP, via anti-mannan Ab. Therefore, a strategy was developed to inhibit activation of the CP in order to allow solely the activation of the LP by immobilized mannan, by using inhibitory anti-C1q antibodies.

Anti-C1q antibodies were tested for their ability to inhibit the CP of complement using immobilized IgM as a specific activator of the CP. Incubation of 1 % normal human serum (NHS) on immobilized IgM induces deposition of C4, which could be dose-dependently inhibited by the anti-C1q mAb 2204, by rabbit IgG anti-C1q antibodies and by Fab fragments prepared from this rabbit anti-C1q antibody preparation. Complete inhibition was reached when the antibodies were applied at 5 µg/ml. In contrast, rabbit IgG prepared from non-immunized rabbits did not have an effect on C4 activation via the CP. These antibodies were tested for their effect on complement activation induced by immobilized mannan. Incubation of NHS on mannan induced a dose-dependent deposition of C4, with a maximal activation at a serum concentration of 1 %. Addition of a fixed concentration of mAb 2204, Fab anti-C1q fragments, or normal rabbit IgG as a control had a slight inhibitory effect on C4 activation. In contrast, rabbit IgG anti-C1q Ab induced complete inhibition of C4 activation by mannan, most likely due to complement consumption via C1q-anti-C1q complexes. These data show that C1q-inhibitory antibodies can block CP activation completely whereas mannan-induced activation of the LP can proceed in a C1q-independent way.

To further examine the role of C1q in complement activation by mannan and by IgM, NHS was depleted from C1q. Depletion of C1q from NHS resulted in a complete inhibition of C4 activation by immobilized IgM (Fig. 3A), as previously described (Fredrikson et al., 1993), whereas C4 activation by immobilized mannan was slightly inhibited by depletion of C1q (Fig. 3B).

Reconstitution of C1q-depleted serum with purified C1q resulted in a complete restoration of C4 activation by IgM (Fig. 3C). In contrast, C4 activation by mannan was slightly inhibited by addition of purified C1q to C1q-depleted serum, possibly due to the presence of an inhibitory protein co-isolated with C1q. The contribution of C1q and MBL to C4 activation by IgM and mannan was further studied using blocking mAb against C1q and MBL, respectively (Fig. 3D). C4 activation on IgM-coated plates was completely inhibited by mAb anti-C1q and no inhibition occurred with a blocking anti-MBL mAb. In contrast, C4 activation induced by mannan was partially inhibited by mAb anti-C1q and strongly inhibited by mAb anti-MBL. Complete inhibition of mannan-induced C4 activation was achieved when a combination of mAb anti-C1q and mAb anti-MBL was used. Together, these data indicate that IgM-mediated activation of C4 is completely dependent on C1q and does not involve MBL. In contrast, mannan-induced activation of C4 is mainly mediated by the LP but comprises a minor contribution of the CP. The latter contribution of the CP can be inhibited by C1q-blocking Ab, thus allowing activation of the LP only.

Complement activation and formation of C5b-9 via the CP and via the LP

The complement activation cascade was further studied using mAb to detect binding of specific complement components upon their activation via the CP and the LP, respectively. Incubation of NHS on immobilized IgM resulted in a dose-dependent deposition of C1q, C4, C3, and C5b-9 to the plate (Fig. 4A). Binding of C1q and subsequent complement activation induced by IgM could be completely inhibited by mAb 2204. Incubation of NHS on immobilized mannan resulted in dose-dependent binding of C4, C3 and C5b-9, whereas binding of C1q was hardly detectable (Fig. 4B). Complement activation by mannan was only slightly inhibited by addition of mAb 2204. Therefore, addition of mAb 2204 in serum allows the specific detection of LP activation using mannan as a ligand, without interference of the CP.

Activation of the alternative pathway

To enable the detection of all complement activation pathways in one assay system, we also studied activation of the alternative pathway in an ELISA system. In contrast to the LP and the CP, activation of the AP is calcium-independent. Therefore, a calcium-free buffer was used, thus excluding involvement of the CP and the LP. As previously described (Fredrikson et al., 1993), incubation of NHS in a buffer containing EGTA and Mg^{++} on plates coated with LPS resulted in a dose-dependent deposition of C3 (Fig. 5A). Some activation of C3 was also observed on plates coated with BSA only, most likely due to spontaneous activation of the AP. Surprisingly, strong activation of C3 was also observed when NHS was incubated on mannan-coated plates using the same conditions, suggesting that mannan may also support activation of the AP (Fig. 5A). Detection of C3 was reduced until background levels when EDTA was present in the complement source (not shown). As expected from an AP-dependent mechanism, C3 activation in calcium-free buffers required a serum concentration that is about 10-fold higher than that required for C3 activation by mannan in a calcium-containing buffer via the LP (compare Fig. 5A with Fig. 4B). Although C3 activation was clearly detectable in a calcium-free buffer, no activation of C4 could be established (Fig. 5B), suggesting that under these conditions activation of C3 is independent of MBL binding and C4 activation.

MBL genotyping by oligonucleotide ligation assay

Single nucleotide polymorphisms in exon 1 of the MBL gene are the most important genetic modifiers of MBL function. We developed three oligonucleotide ligation assays (OLA) for the detection of MBL exon 1 SNP's at codon 52, codon 54, and codon 57, respectively. Using this technique, the presence of B (codon 54), C (codon 57), and D alleles (codon 52), as indicated by formation of double-labeled DNA products using the mutant oligonucleotides, can be easily

detected with a standard laboratory equipment, both in homozygous and in heterozygous patterns (Fig. 6).

Lectin pathway activation is dependent on the MBL genotype

In the Caucasian population, the B allele is the most frequent exon 1 polymorphism in the MBL gene. It has been previously reported that recombinant MBL with the BB genotype has a strongly reduced ability support complement activation. Using MBL genotyping by OLA, sera from individuals with different MBL genotypes were identified. We compared activity of the CP and the LP in serum obtained from an MBL wildtype donor (AA genotype) with serum from donors with a heterozygous and a homozygous mutation at codon 54 (AB and BB genotype, respectively). Serum from all three donors showed strong activation of C4, C3 and C5b-9 via the CP upon incubation on immobilized IgM, in a similar dose-response relationship. In parallel, LP activity was assessed in the same sera by their incubation on immobilized mannan, in the presence of mAb 2204 to block the CP. In sharp contrast to the results obtained on coated IgM, only AA serum, but not BB serum nor AB serum, was able to activate C4, C3 and C5b-9 via the LP. These results clearly indicate that LP activity is strongly determined by the MBL genotype.

Discussion

In the present study, we describe a novel assay for the detection of functional activity of the LP of complement. The assay is based on the detection of various stages of complement activation induced by binding of MBL to immobilized mannan, and involves the addition of inhibitory anti-C1q antibodies to prevent interference of activation of the CP. We demonstrate that in this novel assay system activation of autologous C4, C3, and C5b-9 in full human serum is totally dependent on the presence of functionally active MBL.

Our results show the broad presence of anti-mannan antibodies in the human population. These antibodies may be produced in response to a previous yeast contact and/or may belong to the so-called natural antibodies. Increased levels of antibodies binding to mannan from Saccharomyces Cerevisiae have been described in patients with inflammatory bowel disease (Quinton et al., 1998; Conrad et al., 2002). Certain anti-carbohydrate antibodies can be present in extremely high levels, as is the case for antibodies directed against the major xenoantigen Galα1-3Gal (Galili, 2001). IgG and IgM anti-carbohydrate antibodies can activate the classical complement pathway, and this mechanism is likely to contribute to anti-microbial defense, in addition to lectin-mediated mechanisms. Indeed it has been described that IgG anti-mannan antibodies contribute to opsonization of Candida albicans with C3 (Zhang et al., 1997). Such a mechanism may especially be important in cases where the function of the lectin pathway of complement is impaired. In our study, we were not able to detect any significant difference in levels of anti-mannan Ab between MBL-wildtype and MBL-mutant individuals (not shown).

The presence of highly variable amounts of anti-mannan Ab in human serum necessitates a special strategy to prevent involvement of CP activity in mannan-based assays for LP activation. The present study therefore includes a specific inhibitor of C1q in the assay, which prevents any activation of the CP in serum. Until now, at least two other groups reported a functional assay for the MBL pathway activity that excluded the interference of the CP. Petersen et al. reported an

elegant assay that detects the functional activity of the MBL-MASP complex in serum (Petersen et al., 2001a). This assay is based on the difference between the C1 complex and the MBL-MASP complex with respect to its sensitivity to ionic strength. By addition of 1 M NaCl to the serum dilution buffer, C1q binding and CP activation can be completely prevented whereas MBL binding can proceed. In the assay described by Petersen et al. (Petersen et al., 2001a), the serum incubation step is performed at 4 °C, thus allowing binding of the MBL-MASP complex but not the subsequent complement activation. Activity of the complex is subsequently assessed by addition of exogenous purified C4. The advantage of this technique is that activity of the MBL-MASP complex is directly detected, without any interference of other variables in donor serum. The major difference with the technique as described in the present study is that we now describe a LP assay that assesses activation of autologous complement, which is more representative for the in vivo situation. Furthermore, our assay enables the detection of the complete complement activaton cascade, up to the formation of the membrane attack complex. In this respect, our assay is comparable to hemolytic assays (CH50, AP50) generally used in clinical practice for the evaluation of CP and AP activity. The functional analysis of all three complement activation pathways in parallel by ELISA, as described in the present study, is potentially useful in routine diagnostic laboratories for a more complete diagnostic evaluation of complement defects.

An alternative functional MBL pathway assay was recently described by Zimmerman-Nielsen et al. (Zimmermann-Nielsen et al., 2002). This assay also includes 1 M NaCl in the incubation buffer, but analyzes autologous C4 activation. However, activation of C4 in the presence of 1M NaCl is highly inefficient (Petersen et al. (Petersen et al., 2001a) and our own unpublished observations), which is apparent from the low serum dilutions used in this study. These suboptimal conditions may have a differential effect on C4 activation in serum from various donors, and therefore the C4 activation assessed in this respect is difficult to interpret. Furthermore, also in this assay it is not possible to assess complement activation at a later stage than C4, since formation of

C4b2a is strongly dependent on ionic strength (Laich and Sim, 2001), and accordingly C3 activation is undetectable in 1 M NaCl (our unpublished observations).

An alternative approach for the analysis of LP activity in serum is the quantification of serum-induced hemolysis of mannan-coated sheep erythrocytes (Matsushita and Fujita, 1992; Suankratay et al., 1998). MBL is able to bind to these coated erythocytes (Suankratay et al., 1998), leading to complement activation and erythrocyte lysis. Also in such an hemolytic assay, it is important to prevent interference of the classical complement pathway, which may occur both by anti-mannan antibodies and by anti-erythrocyte antibodies. Suankratay et al. described a method in which mannan-coated erythrocytes were pre-sensitized with purified MBL, followed by incubation with serum in the presence of MgEGTA (Suankratay et al., 1998). This hemolytic assay analyzes the activity of the lectin pathway of complement most likely from C4 until C9, and hence does not provide information about the activity of the MBL-MASP complex in the serum source. Therefore, this assay can not be used to detect a functional impairment of LP activity at the level of MBL. We did not succeed to set up an hemolytic assay with mannan-coated erythocytes using full serum and a C1q inhibitor, probably due to unsufficient sensitivity (data not shown).

We show in the present study that both C1q and MBL have a contribution in the activation of complement by mannan-coated ELISA plates, using inhibitory antibodies directed against C1q and MBL. It is likely that the relative contribution of C1q is strongly increased in donor serum containing high levels of IgG and IgM anti-mannan antibodies in combination with low levels of functional MBL. In such a situation, the contribution of C1q may mask the detection of deficiency of the LP unless CP activation is prevented. Therefore, inhibition of CP is crucial for a reliable functional LP assay.

Different strategies are conceivable for the inhibition of C1q-mediated complement activation in human serum. In the present study, we show C1q inhibition with mAb 2204, an anti-C1q monoclonal antibody that binds to the globular heads of C1q and blocks the interaction with

immunoglobulins. Furthermore, Fab fragments from polyclonal rabbit anti-C1q antibodies, but not complete IgG, can be used to specifically inhibit CP activation. An alternative option is the use of C1q-inhibitory peptides (Roos et al., 2001b). This option is under investigation in our laboratory.

Our studies indicate that incubation of low serum concentrations on immobilized mannan may also activate the alternative pathway. This may involve stabilization of spontaneously activated C3, in a complex with activated factor B and properdin, by its binding to mannan, in a similar way as is effected with other heavily glycosylated microbial products that activate the AP, such as LPS and zymosan. Activation of C3 in the absence of calcium, as well as the lack of detectable C4 activation, strongly suggest an MBL-independent mechanism. Additional studies indicated that binding of MBL in the presence of EGTA was undetectable (Roos et al., 2001a). However, we can not totally exclude that small amounts of MBL binding under these conditions may trigger complement activation, which is subsequently strongly amplified by the alternative pathway at the level of C3. Since activation of the AP requires a high serum concentration, it is highly unlikely that similar MBL-independent complement activation is involved in the LP activity assay when serum is diluted at least 50-fold. This is clearly demonstrated in experiments showing that activation of C4, C3, and C5b-9 was completely undetectable in serum from an individual with a homozygous mutation at codon 54 of the MBL-gene (BB genotype), although this serum had an intact AP activity.

Two members of the ficolin family, L-ficolin and H-ficolin (Hakata antigen) have been recently shown to interact with MASP proteins, and thereby activate complement via the lectin pathway (Matsushita et al., 2000a; Matsushita et al., 2002). Ficolins are multimeric proteins with a lectin domain. L-ficolin does not bind to mannan and is therefore not likely to be involved in complement activation induced by mannan. Both L-ficolin and H-ficolin are present in human serum. At present, there is no information available about the activity of ficolin-mediated

complement activation in full serum. Development of such an assay is dependent on the identification of ficolin-specific ligands that are able to activate ficolin-MASP complexes.

The activity of the LP in human serum is determined by a number of variables, including the concentration and molecular structure of MBL and MASP proteins, the activity of complement proteins from C4 until C9, as well as the presence and activity of serum inhibitors of complement activation (Petersen et al., 2000; Gulati et al., 2002). The assay we now describe enables the functional detection of important consequences of LP activation, i.e. opsonization of the target with complement components, and formation of the membrane attack complex. Until now, various studies have shown that recombinant mutated MBL molecules have an impaired functional activity. Primarily in heterozygous individuals, the consequences of such mutations may be highly variable, depending on the relative expression of the mutated and the wildtype gene. Therefore, functional assessment of LP activity most likely provides a more relevant marker for LP defects than analysis of mutations in in the MBL gene and promoter region. Further studies are now underway to examine the relation between the different parameters involved in LP function and the resulting LP-mediated complement activation.

Acknowledgments

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Legends

Figure 1. Anti-mannan-antibodies in human serum. A-C: Different concentrations of human serum from three different healthy donors were incubated on plates coated with either mannan (closed symbols, solid lines) or BSA (open symbols, dashed lines). Binding of IgG (A), IgA (B) or IgM (C) was detected. As a positive control, plates were incubated with pooled immunoglobulin, as indicated. **D:** Anti-mannan antibodies of the three major Ig classes were quantified in healthy donor serum (N = 70). Solid lines indicate the median concentrations, dashed lines indicate the detection limits.

Figure 2. The effect of C1q inhibitory antibodies on complement activation via the CP and the LP. A: Normal human serum (diluted 1/100) was pre-incubated in the presence or absence of different antibody preparations (rabbit IgG anti-C1q, rabbit Fab anti-C1q, normal rabbit IgG or mAb 2204 in concentrations as indicated), and added to IgM-coated plates to assess CP activation. Activation of C4 was detected. **B:** Normal human serum in different concentrations was pre-incubated in the presence or absence of a fixed concentration of antibodies (rabbit IgG anti-C1q, rabbit Fab anti-C1q, normal rabbit IgG (10 µg/ml) or mAb 2204 (20 µg/ml)). Activation of C4 via the LP was assessed following addition of the mixture to mannan-coated plates.

Figure 3. The role of C1q in activation of the CP and the LP. A, B: Normal human serum or C1q-depleted serum (C1qD-NHS), diluted in GVB++, was incubated on plates coated with IgM (A) and mannan (B), respectively, followed by detection of C4 binding. C: NHS and C1q-depleted NHS (diluted 1/400) were incubated on plates coated with IgM or mannan in the presence or absence of purified C1q (0.5 µg/ml), as indicated. D: NHS was incubated on IgM- or mannan-coated plates in

the presence or absence of blocking mAb directed against MBL (mAb 3F8, 10 μg/ml) or C1q (mAb 2204, 20 μg/ml), or both (combination).

Figure 4. Complement activation via the LP and the CP. Complement activation was induced by incubation of different concentrations of NHS on plates coated with IgM for CP activation (A) or with mannan for LP activation (B), in the presence or absence of mAb 2204 (20 μg/ml). Activation and binding of complement was demonstrated by detection of C1q, C4, C3, and C5b-9 using specific mAb.

Figure 5. Activation of the alternative pathway. NHS was incubated on plates coated with mannan, LPS, or BSA, in a calcium-free buffer (GVB/MgEGTA) to block activation of the CP and the LP. Binding of C3 (A) and C4 (B) was subsequently assessed.

Figure 6. Detection of MBL mutant alleles by oligonucleotide ligation assay. DNA from individuals with different MBL genotypes was used for detection of wildtype or mutant alleles in OLA's for codon 54, codon 57 and codon 52. Results are expressed as the OD at 415 nm of the detection of biotin and dig-labeled OLA products upon ligation using either the wildtype or the mutant allelic probes, and represent mean ± SD of one out of three experiments performed in duplicate. MBL genotypes were confirmed by DNA sequencing.

Figure 7. Lectin pathway activity is impaired in serum from MBL-mutant donors. Serum was obtained from three different donors with a wildtype MBL genotype (AA), a heterozygous mutation (AB) or a homozygous mutation (BB) at codon 54. Different concentrations were applied to plates coated with IgM to assess CP activity, in the absence of mAb 2204 (A), or with mannan to assess LP activity, in the presence of mAb 2204 (B). Deposition of C4, C3, and C5b-9 was assessed.

Table 1. Oligonucleotides used for MBL genotyping

Oligonucleotide	Sequence
PCR forward	5'-ACCCAGATTGTAGGACAGAG-3'
PCR reverse	5'-GTTGTTGTTCTCCTGTCCAG-3'
OLA 52-common	5'-P-CCCATCTTTGCCTGG-bio-3'
OLA 52-wildtype	5'-dig-CCTTGGTGCCATCACG-OH-3'
OLA 52-mutant	5'-dig-CCCTTGGTGCCATCACA-OH-3'
OLA 54-common	5'-P-CATCACGCCCATCTTTG-bio-3'
OLA 54-wildtype	5'-dig-CTTTTCTCCCTTGGTGC-OH-3'
OLA 54-mutant	5'-dig-CCTTTTCTCCCTTGGTGT-OH-3'
OLA 57-common	5'-P-CCTTGGTGCCATCACG-bio-3'
OLA 57-wildtype	5'-dig-TGGTTCCCCCTTTTCTC-OH-3'
OLA 57-mutant	5'-dig-CTGGTTCCCCCTTTTCTT-OH-3'

PCR primers were derived from (Garred et al., 1996) with slight modifications. OLA primers are labelled with either biotin (bio) or digoxygenin (dig). All primers were obtained from Eurogentec (Seraing, Belgium).

CLAIMS

1. A method of functionally determining deficiencies in the complement system by only assaying the classical pathway, the alternative pathway, or the lectin pathway, respectively, the method comprising the steps of

(a) providing a sample of mammalian blood;

(b) selecting one pathway to be assayed;

(c) preventing in the sample the activation of the two non-assayed pathways;

(d) activating in the sample the pathway to be assayed; and

(e) determining in the sample any activation of the common terminal complement pathway.

2. The method as in claim 1, wherein under (c) the activation of the classical pathway is prevented by providing in the sample antibodies against C1q proteins of the complement system.

3. The method as in claim 1, wherein under (c) the activation of the classical pathway is prevented by providing in the sample peptides inhibiting C1q proteins of the complement system.

4. The method as in claim 1, wherein under (c) the activation of the classical pathway is prevented by providing in the sample antibodies against immunoglobulins IgG and/or IgM.

5. The method as in claim 1, wherein under (c) the activation of the classical pathway is prevented by providing in the sample a Ca^{2+} or Mg^{2+} binding agent.

6. The method as in claim 5, wherein the binding agent is a chelating agent.

7. The method as in claim 1, wherein under (c) the activation of the alternative pathway is prevented by dilution of the sample.

8. The method as in claim 1, wherein under (c) the activation of the alternative pathway is prevented by

providing in the sample a protease inhibitor to factor D of the complement system.

9. The method as in claim 1, wherein under (c) the activation of the lectin pathway is prevented by providing in the sample a cell surface carbohydrate.

10. The method as in claim 9, wherein the cell surface carbohydrate is a mannan.

11. The method as in claim 1, wherein under (d) the classical pathway is activated by providing in the sample immunoglobulins IgG and/or IgM.

12. The method as in claim 1, wherein under (d) the alternative pathway is activated by providing in the sample lipopolysaccharides or derivatives thereof.

13. The method as in claim 1, wherein under (d) the lectin pathway is activated by providing in the sample mannan binding proteins.

14. The method as in claim 1, wherein under (e) any activation of the common terminal complement pathway in the sample is determined by determining the activity of a complement protein from C4 to C9 of the same.

15. The method as in claim 14, wherein any activation is determined by providing in the sample antibodies against formed C5b-9 complex.

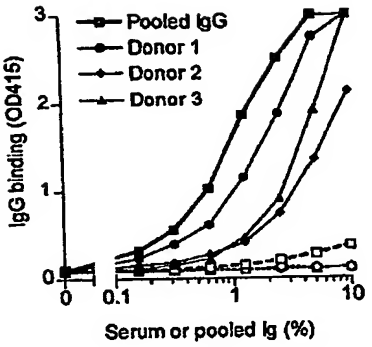
Abstract

Mannan-binding lectin (MBL) is a major initiator of the lectin pathway (LP) of complement. Polymorphisms in exon 1 of the MBL gene are associated with impaired MBL function and infections. Functional assays to assess the activity of the classical pathway (CP) and the alternative pathway of complement in serum are broadly used in patient diagnostics. We now developed a functional LP assay that enables the specific quantification of autologous MBL-dependent complement activation in human serum.

Complement activation was assessed by ELISA using coated mannan to assess the LP and coated IgM to assess the CP. However, normal human serum contains IgG, IgA and IgM antibodies against mannan, as shown by ELISA. These antibodies are likely to induce CP activation. Using C1q-blocking and MBL-blocking mAb, it was confirmed that both the LP and the CP contribute to complement activation by mannan. In order to quantify LP activity without the interference of the CP, LP activity was measured in serum in the presence of C1q-blocking Ab. Activation of serum on coated IgM via the CP resulted in a dose-dependent deposition of C1q, C4, C3, and C5b-9. This activation and subsequent complement deposition was completely inhibited by the C1q-blocking mAb 2204 and by polyclonal Fab anti-C1q Ab. Evaluation of the LP in the presence of mAb 2204 showed a strong dose-dependent deposition of C4, C3, and C5b-9 using serum from MBL-wildtype (AA) but not MBL-mutant donors (AB or BB genotype), indicating that complement activation under these conditions is MBL-dependent and C1q-independent. Donors with different MBL genotypes were identified using a newly developed oligonucleotide ligation assay for detection of MBL exon 1 polymorphisms.

We describe a novel functional assay that enables quantification of autologous complement activation via the LP in full human serum up to the formation of the membrane attack complex. This assay offers novel possibilities for patient diagnostics as well as for the study of disease association with the LP.

A



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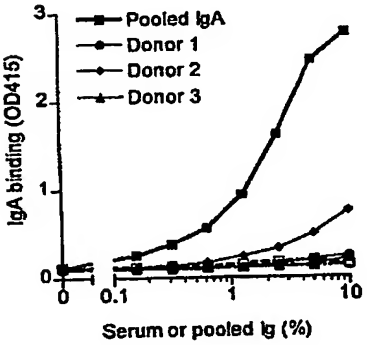

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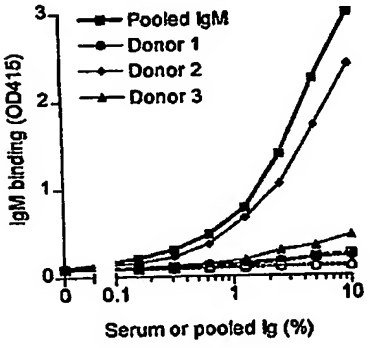

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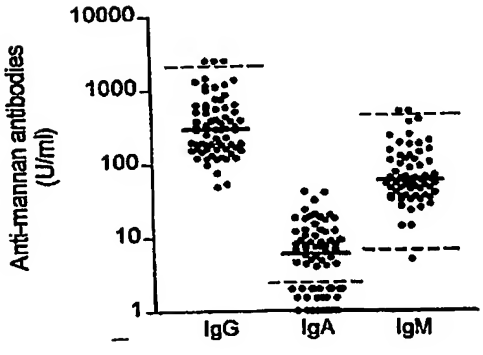
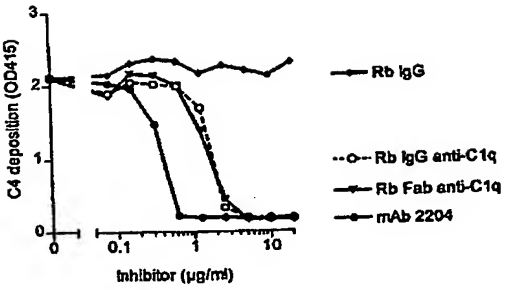
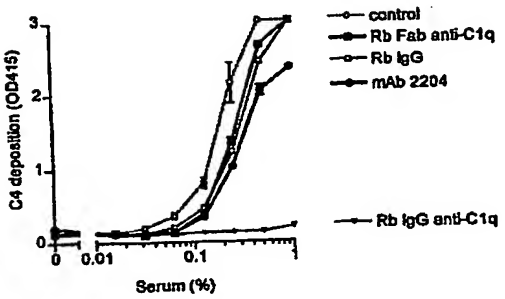
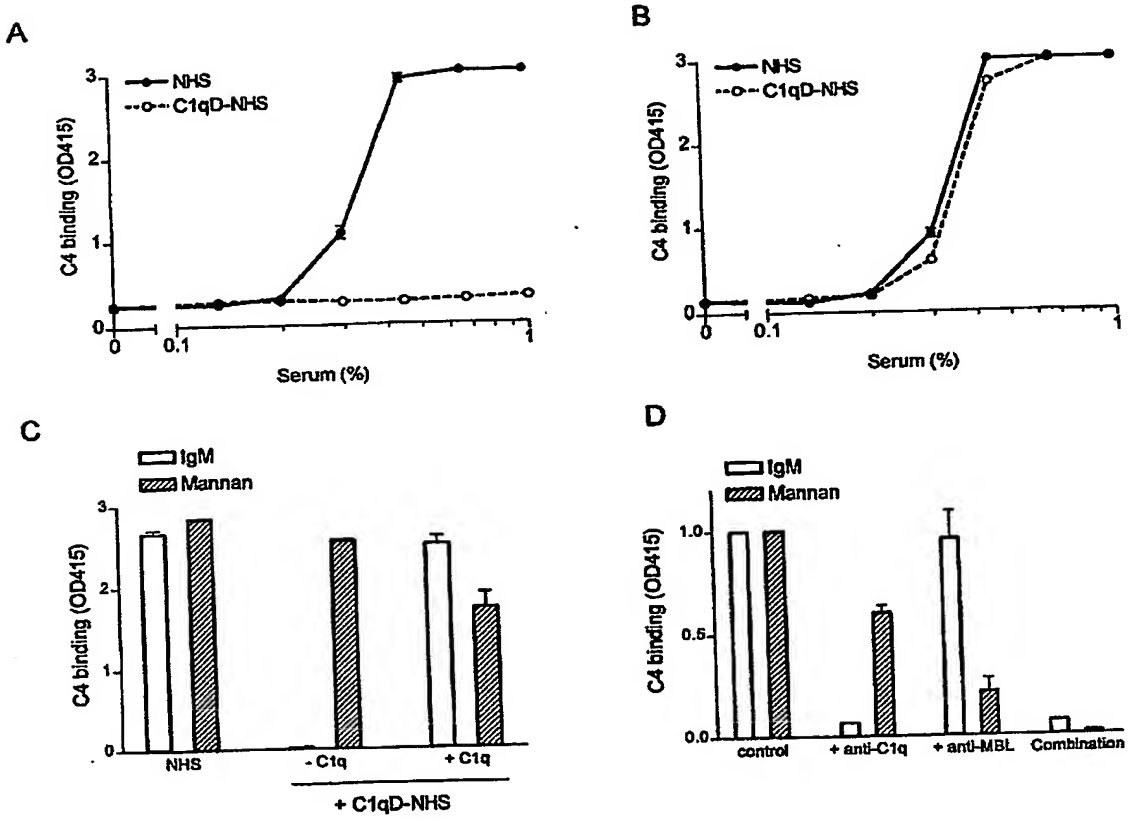
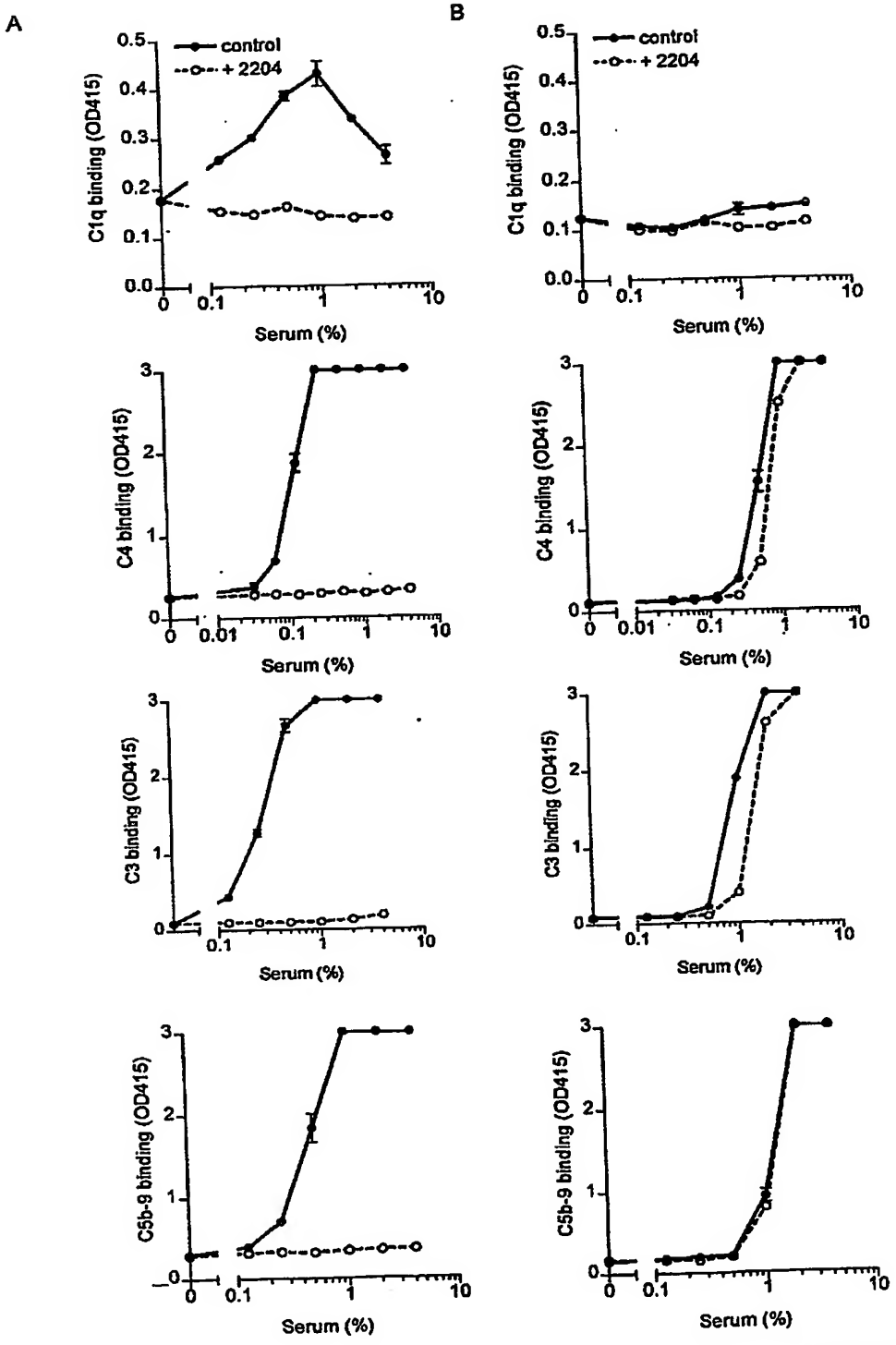




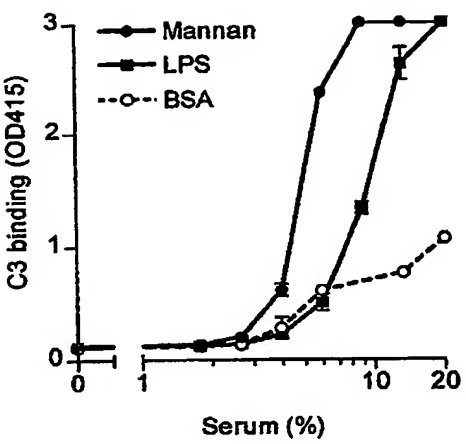


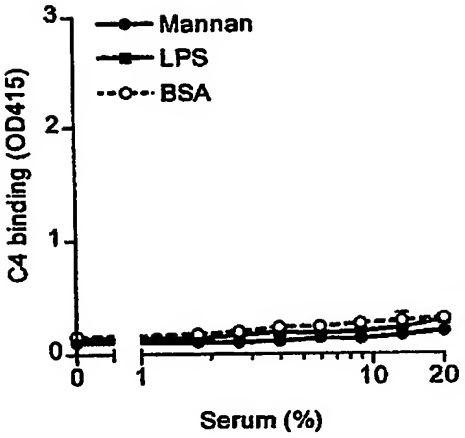
A. Roos et al. Figure 2


A
NHS
C1qD-NHS
C4 binding (OD415)
Serum (%)
B
NHS
C1qD-NHS
C4 binding (OD415)
Serum (%)
C
IgM
Mannan
C4 binding (OD415)
NHS
- C1q
+ C1q
+ C1qD-NHS
D
IgM
Mannan
C4 binding (OD415)
control
+ anti-C1q
+ anti-MBL
Combination

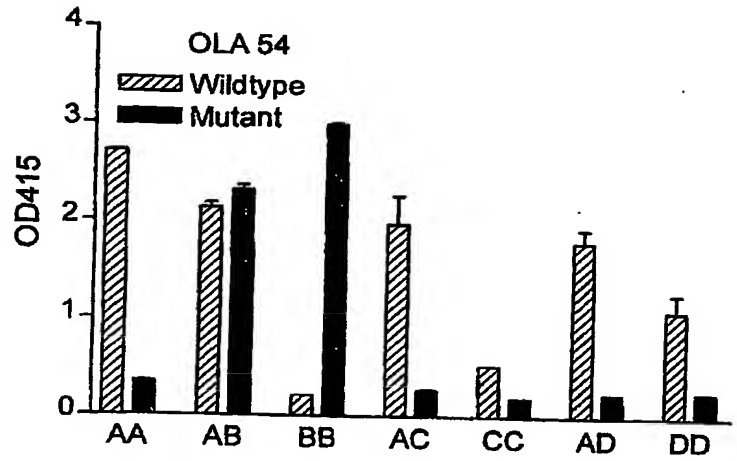


A. Roos et al. Figure 4





A. Roos et al. Figure 5


OLA 54
Wildtype
Mutant
OD415
4
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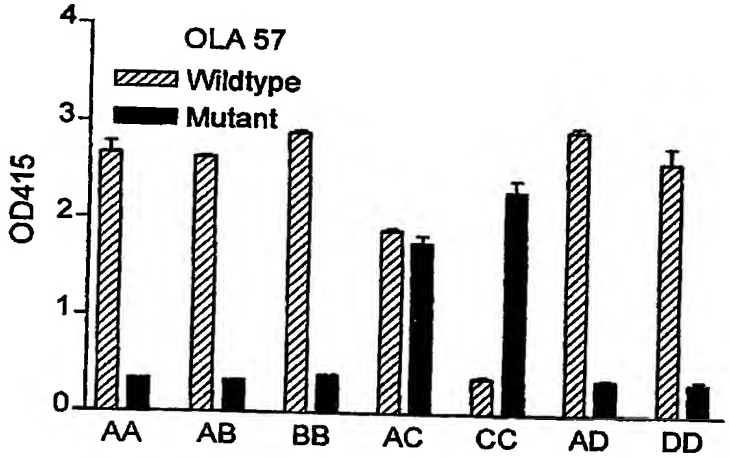
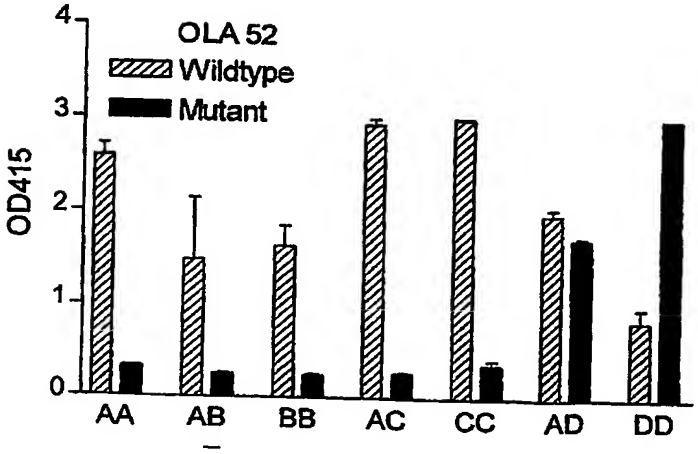
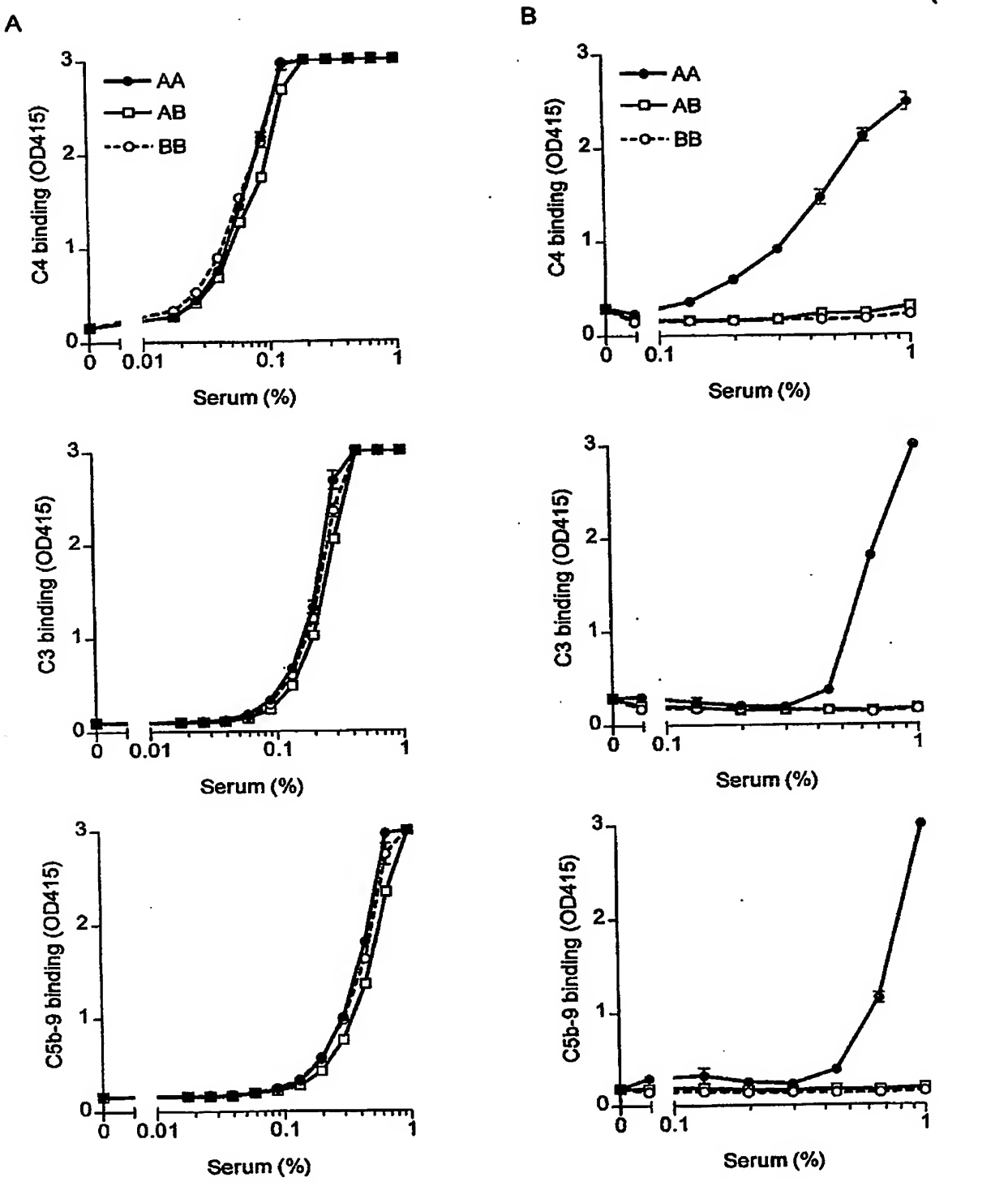
AC
CC
AD
DD


OLA 57
Wildtype
Mutant
OD415
4
3
2
1
0
AA
AB
BB
AC
CC
AD
DD


OLA 52
Wildtype
Mutant
OD415
4
3
2
1
0
AA
AB
BB
AC
CC
AD
DD


A
B
AA
AB
BB
C4 binding (OD415)
C3 binding (OD415)
C5b-9 binding (OD415)
Serum (%)
0
0.01
0.1
1
2
3